**STIC-ILL**

**From:** Goldberg, Jeanine
**Sent:** Tuesday, October 22, 2002 4:22 PM
**To:** STIC-ILL
**Subject:** please pull mt damage

1. JOURNAL OF CARDIOVASCULAR PHARMACOLOGY, (1996) 28 Suppl 1 S1-10. Ref: 80
Journal code: 7902492. ISSN: 0160-2446.

2. Circulation, (10/21/97, 1997) Vol. 96, No. 8 SUPPL., pp. I604.
Meeting Info.: 70th Scientific Sessions of the American Heart Association Orlando, Florida, USA November 9-12, 1997
ISSN: 0009-7322.

3. AMERICAN JOURNAL OF OTOLOGY, (2000 Mar) 21 (2) 161-7.
Journal code: 7909513. ISSN: 0192-9763.

4. CIRCULATION RESEARCH, (2000 May 12) 86 (9) 960-6.
Journal code: 0047103. ISSN: 1524-4571.

5. CIRCULATION, (2002 Feb 19) 105 (7) 849-54.
Journal code: 0147763. ISSN: 1524-4539.

6. Mitochondrial DNA Mutations in Aging, Disease and Cancer (1998), 239-263.
Editor(s): Singh, Keshav K. Publisher: Springer, Berlin, Germany.
CODEN: 67RGAO
DT Conference; General Review.

7. BIOCHIMICA ET BIOPHYSICA ACTA, (1996 Jul 18) 1275 (1-2) 10-5. Ref: 48
Journal code: 0217513. ISSN: 0006-3002.

8.

Jeanine Enewold Goldberg
1634
CM1--12D11
Mailbox-- 12E12
306-5817

# Cigarette Smoke Exposure and Hypercholesterolemia Increase Mitochondrial Damage in Cardiovascular Tissues

Cynthia A. Knight-Lozano, BS; Christal G. Young, BS; David L. Burow, MS; Zhao Yong Hu, MD; Dale Uyeminami, BS; Kent E. Pinkerton, PhD; Harry Ischiropoulos, PhD; Scott W. Ballinger, PhD

***Background***—A shared feature among cardiovascular disease risk factors is increased oxidative stress. Because mitochondria are susceptible to damage mediated by oxidative stress, we hypothesized that risk factors (secondhand smoke and hypercholesterolemia) are associated with increased mitochondrial damage in cardiovascular tissues.

***Methods and Results***—Atherosclerotic lesion formation, mitochondrial DNA damage, protein nitration, and specific activities of mitochondrial proteins in cardiovascular tissues from age-matched C57 and apoE$^{-/-}$ mice exposed to filtered air or secondhand smoke were quantified. Both secondhand smoke and hypercholesterolemia were associated with significantly increased mitochondrial DNA damage and protein nitration. Tobacco smoke exposure also resulted in significantly decreased specific activities of mitochondrial enzymes. The combination of secondhand smoke and hypercholesterolemia resulted in increased atherosclerotic lesion formation and even greater levels of mitochondrial damage.

***Conclusions***—These data are consistent with the hypothesis that cardiovascular disease risk factors cause mitochondrial damage and dysfunction. (***Circulation.*** **2002;105:849-854.)**

**Key Words:** atherosclerosis ■ smoking ■ hypercholesterolemia ■ mitochondria

Although there is considerable controversy about the precise sequence of events leading to cardiovascular disease (CVD), growing evidence indicates that atherosclerotic lesions result from oxidative stress mediated by metabolic defects and environmental insults. Secondhand smoke (SHS) is considered a risk factor in adults and children and has been linked with numerous vascular effects, decreased levels of serum antioxidants (ie, vitamins E and C), increased products of lipid peroxidation, platelet aggregation, and accelerated atherogenesis.[1-9] More than 400 000 people in the United States die from tobacco smoke–related illnesses each year, with the largest portion being cardiovascular related (≈190 000 CVD deaths per year are related to cigarette smoke; 37 000 to 40 000 of these deaths are attributable to SHS exposure).[2] Although it is known that SHS exposure causes a multitude of effects, ranging from a reduction in arterial oxygen-carrying capacity by increasing serum carboxyhemoglobin levels to increased endothelial cell injury and altered cardiac cellular metabolism,[10,11] the specific events contributing to CVD development are not fully characterized and understood.

A shared feature among CVD risk factors is the generation of increased reactive oxygen and nitrogen species (ROS and RNS, respectively), which can cause mitochondrial injury in vitro, ranging from mtDNA damage and decreased adenine nucleotide translocator (ANT) activity to alteration of mitochondrial proteins.[12-14] While the mtDNA encodes genes essential for oxidative phosphorylation, the ANT enzyme is responsible for translocation of adenine nucleotides across the inner mitochondrial membrane, and, thus, both are essential for oxidative phosphorylation and ATP production. In addition, mitochondria play a role in cell signaling and apoptosis.[15,16] Consequently, increased vascular oxidative stress may cause mitochondrial damage that potentially impacts a variety of cellular functions, ranging from energy production to cell signaling. Mouse models of hypercholesterolemia and SHS exposure were used to determine whether a relationship exists between SHS, hypercholesterolemia, and mitochondrial damage in cardiovascular tissues. In these studies, atherosclerotic lesion formation, protein nitration levels, mtDNA damage, mitochondrial superoxide dismutase (SOD2), and ANT-specific activities were quantified in cardiovascular tissues from age-matched normocholesterolemic and hypercholesterolemic mice exposed to filtered air or SHS.

Received August 22, 2001; accepted December 19, 2001.

From the Division of Cardiology (C.A.K., C.Y.G., D.L.B., Z.H., S.W.B.), University of Texas Medical Branch, Galveston, Tex; Institute of Toxicology and Environmental Health (D.U., K.E.P.), University of California, Davis; and Children's Hospital of Philadelphia (H.I.), Abramson Pediatric Research Center, Philadelphia, Pa.

Correspondence to S.W. Ballinger, PhD, Department of Internal Medicine, Division of Cardiology, University of Texas Medical Branch, Galveston, TX 77555-1064. E-mail swballin@utmb.edu



*Circulation* is available at http://www.circulationaha.org

DOI: 10.1161/hc0702.103977

## Methods

### Mice

C57 and apoE$^{-/-}$ male mice were purchased from Jackson Laboratories (Bar Harbor, Maine). The apoE$^{-/-}$ mouse lacks apolipoprotein E, a high-affinity ligand for lipoprotein receptors, and consequently has elevated levels of serum LDL cholesterol and triglycerides, developing atherosclerotic plaques in a fashion similar to humans.[17,18] The C57BL/6 mouse shares the same genetic background and thus is the proper normocholesterolemic control. All mice were fed diets that contain 4.5% fat by weight (PicoLab Rodent Chow 20) and water ad libitum. Animal care was given in accordance with institutional guidelines.

### SHS Exposure

Exposures were conducted at the Institute of Toxicology and Environmental Health inhalation facilities (University of California, Davis) in accordance with institutional guidelines. Animals (6 weeks old) were acclimated in filtered air chambers for 1 week. Commencing at 7 weeks of age, mice (N=20/genotype per exposure group) were exposed to either 42 days of filtered air; 21 days of filtered air followed by 21 days of SHS; or 42 days of SHS, 6 hours per day, 5 days per week (Monday through Friday), for a total of 252 hours of exposure. A side-stream dose of 30 mg/m$^3$ total suspended particulate (TSP) (equivalent to SHS from 2 cigarettes every 15 minutes, 1R4F standard reference cigarette, Tobacco and Health Research Institute) was used. A dose of 1 mg/m$^3$ TSP was also used to assess aortic mtDNA damage.

### Euthanasia

Animals were anesthetized (IP injection of 40 mg/kg Ketaset and 10 mg/kg xylazine), and tissues were harvested and stored at −80°C (freshly dissected tissues were used for ANT activities and oil red O staining). Because of limited amounts of available tissue, aortic tissues were used to assess atherosclerotic lesion formation and mtDNA damage, whereas heart tissues were used to assess specific enzyme activities and protein nitration.

### Atherosclerotic Lesion Assessment

#### *Aortic Sinus*

Hearts with the aortic root attached were dissected free, fixed in 4% paraformaldehyde, and embedded in paraffin. Serial sections (5 μm) were stained with hematoxylin and eosin and photographed.

#### *Whole Aortas*

Hearts with attached aortas (down to the iliac artery) were dissected free, fixed in 10% phosphate-buffered formalin, and stored in PBS plus 0.1% $NaN_3$ at 4°C. Aortas were cut longitudinally, incubated with oil red O stain, and flattened onto slides using a coverslip to allow visualization of the intima en face. Aortas were photographed using a Nikon FDX 35-mm camera and Nikon U-III photo system, which were mounted onto a Zeiss Stemi dissecting microscope.

### Atherosclerotic Lesion Quantification

Images were scanned into Photoshop (Adobe), and atherosclerotic lesion areas were selected by contrast differences and transported into ScionImage (National Institutes of Health/Scion Corp) to measure the lesion area. Total atherosclerotic lesion area (mm$^2$) was quantified from aortic roots, and oil red O staining of whole aortas was expressed as percent positive staining area relative to total aortic area.

### Cholesterol Determination

Total plasma cholesterol levels were determined using the Infinity (Sigma) cholesterol reagent, per instructions.[19] Sample cholesterol levels were extrapolated from standard curves using known cholesterol standards (Sigma C-0284).

### Mitochondrial SOD2-Specific Activity

SOD2 activity was determined using the cytochrome C reduction assay[20] in the presence of 3 mmol/L potassium cyanide. Control reactions using purified CuZnSOD and SOD2 (Sigma S-2515 and S-5639, respectively) verified that potassium cyanide inhibited CuZnSOD. Specific activity was calculated by dividing SOD2 activity by the amount of SOD2 protein present, determined by immunoblots with SOD2 antibody (Research Diagnostics, Inc).

### ANT-Specific Activity

ANT-specific activity was determined by dividing atractyloside-sensitive $^3$H-ADP uptake[13] by the amount of ANT protein (determined from immunoblots[21]). ANT antibodies were provided by Dr Douglas C. Wallace (Emory University, Atlanta, Ga).

### Protein and SOD2 Nitration

Protein nitration was quantified from immunoblots (30 μg total protein) using 3-nitrotyrosine antibody.[22] To determine SOD2 nitration, whole-heart homogenates were diluted to 1 mg/mL in 1×RIPA buffer (150 mmol/L NaCl, 50 mmol/L Tris, pH 7.5, 1 mmol/L EGTA, 0.25% sodium deoxycholate, 1% NP-40, 1 mmol/L sodium orthovanadate, and 0.05% Triton X-100 and protease inhibitors) and centrifuged at 13 000*g* for 10 minutes. Immunoprecipitations (0.5 mg) of the supernatant were performed with rabbit polyclonal SOD2 antibody (Research Diagnostics). Proteins A and G bound to Sepharose (Pierce Chemical Co) were used to pellet the specific immune complex by centrifugation at 3000*g* for 3 minutes. The immunoprecipitates were separated on 12% SDS-PAGE gels, transferred, and immunoblotted with 3-nitrotyrosine antibody.

### Quantitative Polymerase Chain Reaction for Evaluating mtDNA Damage

Quantitative polymerase chain reaction (QPCR) was performed as previously described[12] using primer pairs M13597 FOR (bps 13597 to 13620) and M13361 REV (bps 13361 to 13337). Copy number differences (mtDNA) were normalized using QPCR of an 80-bp region of the mitochondrial genome that yields products directly related to gene copy numbers (primers M13281F [bps 13281 to 13303] and M13361REV).

### Statistical Analysis

Results are expressed as mean±SEM. Two-way ANOVA (genotype and SHS exposure time) was used in all instances except for analysis of mtDNA damage (3-way ANOVA was used; genotype, SHS time, and dose) to test for the global hypothesis that all samples were drawn from a single population. If this test yielded a significant value ($P<0.05$), a Student-Newman-Keuls test was used for group comparisons.

## Results

### SHS Exposure Increases Atherosclerotic Lesion Formation in apoE$^{-/-}$ Mice

Hematoxylin-eosin staining of aortic sinuses revealed an increase in mean lesion size in SHS-exposed apoE$^{-/-}$ mice compared with unexposed apoE$^{-/-}$ mice (Figure 1, 76% and 156% at 21 and 42 days of SHS, respectively). By contrast, no obvious lesions were observed within the aortic sinus region from the C57 mouse exposure groups. Quantitative assessment of whole aortas (from the aortic root to the iliac artery) by oil red O staining revealed increases in the mean percentage of positive staining area in SHS-exposed apoE$^{-/-}$ compared with unexposed apoE$^{-/-}$ mice (4.5-fold, Figure 2, $P<0.05$). However, aortas from C57 mice exposed to SHS had 2.1- and 3.7-fold increases (21- and 42-day SHS exposures, respectively) in the mean percentages of positive staining compared with unexposed counterparts (Figure 2).


**Figure 1.** Hematoxylin-eosin staining of the aortic sinus from apoE$^{-/-}$ mice exposed to either 42 days of filtered air (unexposed), 21 days of filtered air followed by 21 days of 30 mg/m$^3$ TSP (21 days SHS exposure), or 42 days of 30 mg/m$^3$ TSP (42 days SHS exposure). White arrows indicate obvious lesions. Numbers below represent mean±SEM for lesion area for each group of apoE$^{-/-}$ mice.

As expected, apoE$^{-/-}$ mouse aortas had significantly higher levels of oil red O staining compared with C57 mice ($P<0.05$). The effects of SHS were most pronounced in the apoE$^{-/-}$ mice, consistent with significant interaction between hypercholesterolemia and SHS exposure ($P=0.036$).

Predictably, the apoE$^{-/-}$ mice had significantly higher plasma cholesterol levels compared with the C57 mice overall (587.04±21.97 mg/dL versus 106.05±1.51 mg/dL, apoE$^{-/-}$ versus C57, all groups combined, $P<0.05$). SHS exposure did not significantly change total cholesterol levels among the apoE$^{-/-}$ or C57 mice when compared with unexposed genotype-matched controls (apoE$^{-/-}$, 570.46±38.55 mg/dL [unexposed] versus 584.49±44.57 mg/dL and 606.17±32.52 mg/dL after 21 and 42 days of SHS exposure, respectively; C57, 104.37±3.19 mg/dL [unexposed] versus 107.53±6.18 mg/dL and 106.25±6.54 mg/dL after 21 and 42 days of SHS exposure, respectively), consistent with previous reports.[23] There was no significant interaction between genotype and SHS exposure ($P=0.326$).

## SHS Exposure Significantly Altered Mitochondrial Protein–Specific Activities and Increased Protein Nitration

SHS was associated with a significant decrease in both SOD2- and ANT-specific activities in hearts from apoE$^{-/-}$ and C57 mice ($P<0.05$). Figure 3A shows that SOD2-specific activity was reduced after 21 and 42 days of 30 mg/m$^3$ TSP exposure in apoE$^{-/-}$ and C57 mice, respectively. Similarly, Figure 3B shows that ANT-specific activity was reduced in apoE$^{-/-}$ and C57 mice after 42 days of SHS exposure ($P<0.05$). There was significant interaction between SHS exposure and hypercholesterolemia in decreasing the specific activity of both enzymes (SOD2, $P=0.001$; ANT, $P<0.001$). These data are consistent with the notion that increased oxidative stress reduces mitochondrial SOD and ANT activities.

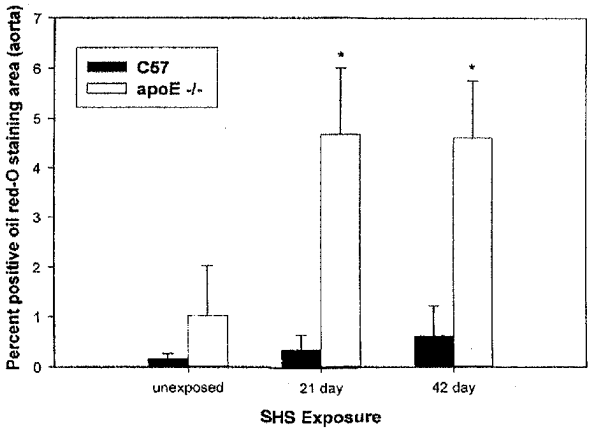


**Figure 2.** Percent oil red O staining area in unexposed, 21-day SHS-exposed, and 42-day SHS-exposed mice. Filled and open bars represent C57 and apoE$^{-/-}$ mice, respectively. *Significant difference exists between exposed and unexposed apoE$^{-/-}$ mice.

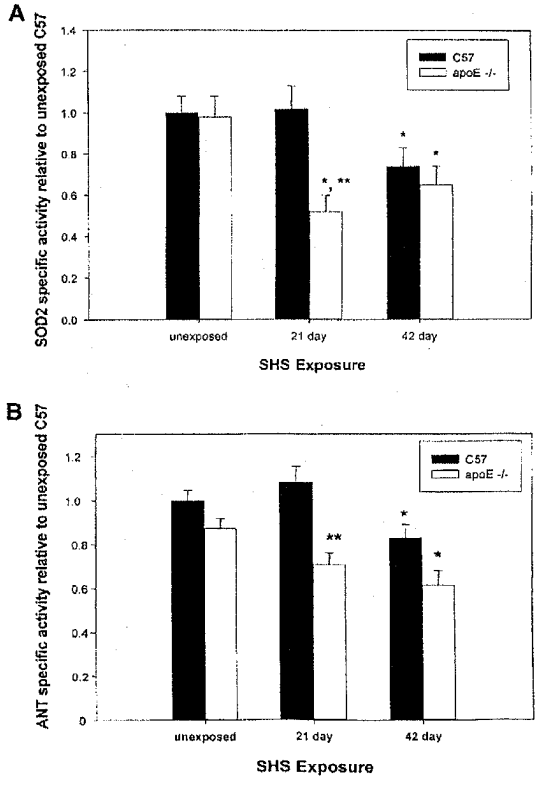


**Figure 3.** Specific enzyme activities in unexposed, 21-day SHS-exposed, and 42-day SHS-exposed mice. A, SOD2-specific activity. B, ANT-specific activity. All activities are expressed relative to the unexposed C57 group. *Significant differences exist between genotype-matched exposed and unexposed groups. **Significant differences exist between apoE$^{-/-}$ and C57 counterparts.

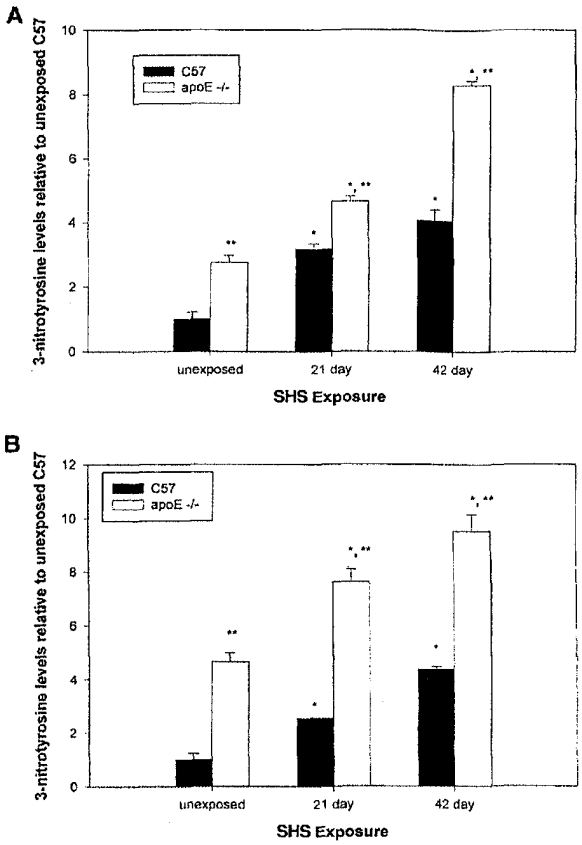


**Figure 4.** 3-nitrotyrosine levels in unexposed, 21-day SHS-exposed, and 42-day SHS-exposed mice. A, 3-Nitrotyrosine levels in protein homogenates prepared from hearts. B, 3-nitrotyrosine levels from SOD2 protein immunoprecipitated from heart homogenates. All 3-nitrotyrosine levels are expressed relative to the unexposed C57 mice. *Significant differences exist between genotype-matched exposed and unexposed groups. **Significant differences exist between apoE$^{-/-}$ and C57 counterparts.

Because previous reports have indicated that SOD2 can be inactivated by tyrosine residue nitration,[14] we assessed 3-nitrotyrosine adduct levels in proteins from SHS-exposed mice. Figure 4A shows that SHS exposure and hypercholesterolemia increased 3-nitrotyrosine levels, and their combination resulted in greater 3-nitrotyrosine levels (interaction between hypercholesterolemia and SHS exposure, $P<0.001$). Similarly, Figure 4B shows that both hypercholesterolemia and SHS exposure independently increased the level of nitrated SOD2 protein in heart tissues and was highest in SHS-exposed apoE$^{-/-}$ mice (significant interaction between hypercholesterolemia and SHS exposure, $P<0.001$). These data are consistent with the hypothesis that risk factors such as hypercholesterolemia and SHS exposure increase protein nitration, contributing to the inactivation of mitochondrial SOD2.

### SHS Exposure and Hypercholesterolemia Increase mtDNA Damage

SHS exposure (30 mg/m$^3$ TSP) resulted in higher levels of aortic mtDNA damage in apoE$^{-/-}$ and C57 mice compared with unexposed animals (Figure 5). Increased duration of exposure significantly increased aortic mtDNA damage in both genotypes of mice ($P<0.001$), and hypercholesterolemia accentuated the effects of SHS exposure (there was significant interaction between hypercholesterolemia and SHS exposure, $P<0.001$). Finally, unexposed apoE$^{-/-}$ mice had significantly greater levels of aortic mtDNA damage compared with their C57 counterparts (Figure 5), suggesting that in addition to SHS exposure, hypercholesterolemia contributed to aortic mtDNA damage ($P=0.013$). To examine the effects of a lower SHS dose, mtDNA damage was assessed in aortas from apoE$^{-/-}$ and C57 mice exposed to the same regimen, but at 1 mg/m$^3$ TSP. The lower-dose SHS exposure was associated with significantly increased aortic mtDNA damage in both apoE$^{-/-}$ and C57 mice after 42 days of exposure (Figure 5), and hypercholesterolemia increased the effects of SHS exposure (42-day apoE$^{-/-}$ versus 42-day C57, $P<0.001$), suggesting that relatively low levels of passive smoke exposure are capable of causing significant mtDNA damage and that hypercholesterolemia accentuates these effects (there was significant interaction between hypercholesterolemia and SHS exposure, $P=0.002$). As expected, mtDNA damage was the greatest at 30 mg/m$^3$ TSP in both genotypes of mice when compared with 1 mg/m$^3$ TSP ($P<0.001$).

## Discussion

There is a growing consensus that increased oxidative stress mediates CVD. However, the biologically seminal events

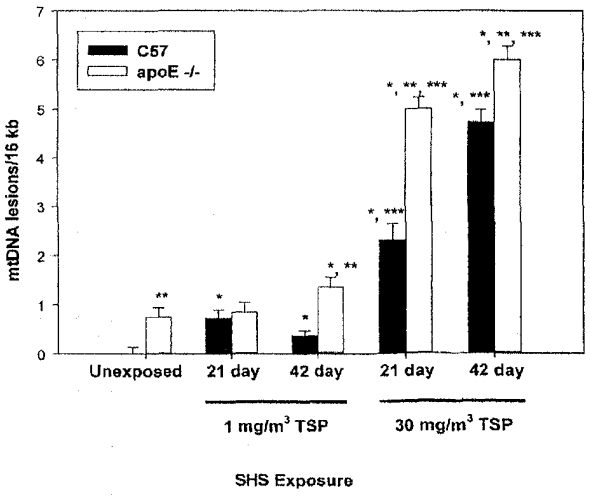


**Figure 5.** MtDNA damage in SHS-exposed mice. MtDNA damage was assessed in whole aortas from unexposed, 21-day SHS-exposed, and 42-day SHS-exposed mice. SHS dose (1 and 30 mg/m$^3$ TSP) is indicated. *Significant differences exist between genotype-matched exposed and unexposed groups. **Significant differences exist between apoE$^{-/-}$ and C57 counterparts. ***Significant differences exist between 30 and 1 mg/m$^3$ TSP counterparts.

initiated by oxidative stress that ultimately result in disease are less clear. By virtue of their susceptibility to oxidative damage as well as their role in energy production and cell signaling, mitochondria are biologically relevant targets for damage mediated by oxidative stress. This study shows that SHS and hypercholesterolemia were individually associated with increased mtDNA damage and protein nitration, whereas SHS exposure was also associated with decreased specific activities of SOD2 and ANT. The combined effects of SHS exposure and hypercholesterolemia resulted in greater levels of mtDNA damage, protein nitration, and decreased specific activities of SOD2 and ANT. These results are consistent with the hypothesis that CVD risk factors cause mitochondrial damage in cardiovascular tissues.

Mitochondrial damage could affect cardiovascular cell function through a variety of mechanisms. In addition to contributing to increased formation of reactive oxygen species and RNS (and thus modulating available ·NO nitric oxide levels), which are capable of oxidizing LDL (a key step in atherogenesis) and mitochondrial proteins, altered antioxidant function and radical production may modify redox signaling pathways mediated through the mitochondrion, potentially influencing cellular regulatory pathways. Chronic mitochondrial damage could also affect cellular energy production; however, because some tissues can have relatively low energetic thresholds (ie, require a minority of properly functioning mitochondria to meet energetic demands), we predict that the initial impact of mitochondrial damage contributes to cell dysfunction via altered signaling and ·NO availability. Chronic oxidant exposure would ultimately result in compromised energy production and cell death as well.

Increased levels of nitration products have been observed in human atherosclerotic tissues,[24] and it has been hypothesized that the relative balance of ·NO and $O_2\cdot^-$ (superoxide) within the vascular environment are important factors in influencing the reactivity of ·NO toward the detrimental effects of RNS formation.[25] We have shown that nitration of SOD2 is increased in apoE$^{-/-}$ mice and that SHS exposure increases these levels in apoE$^{-/-}$ and C57 mice. These data support the concepts that RNS exist intramitochondrially and that they are increased with CVD risk factors. It has been shown that certain mitochondrial proteins, including SOD2, are susceptible to reactive nitrogen-mediated damage and inactivation.[26–28] The observed increase in SOD2 nitration and decrease in SOD2-specific activity associated with both SHS and hypercholesterolemia in this study are consistent with these observations and the notion that CVD risk factors increase mitochondrial damage. Moreover, the observation that SOD2-specific activities were not significantly different between the 42-day SHS-exposed C57 and apoE$^{-/-}$ mice (although they were significantly different from unexposed controls), despite the fact that the SHS-exposed apoE$^{-/-}$ mice had significantly higher levels of 3-nitrotyrosine compared with their C57 counterparts overall, is consistent with reports of differential susceptibility of SOD2 tyrosine residues to nitration. For example, whereas only 3 (Tyr34, Tyr45, and Tyr193) of the 9 total tyrosine residues in the SOD2 subunit seem to be susceptible to nitration, Tyr34 located near the manganese atom in the active site is the most susceptible to nitration and is believed to be the primary residue associated with inactivation of SOD2. The 2 remaining residues are less vulnerable to nitration and seem unrelated to enzyme inactivation. Consequently, the first SOD2 tyrosine residue to be nitrated by RNS is generally the one associated with enzyme inactivation. Whereas nitration of additional tyrosine residues can occur, it will not significantly contribute to decreased specific activity.

Although the mice in this study were relatively young (15 weeks of age), and the overall ANT-specific activities between the unexposed apoE$^{-/-}$ and C57 mice were not different, SHS exposure did result in decreased ANT specific activity in both C57 and apoE$^{-/-}$ mice, consistent with the concept that increased oxidative stress can alter ANT function.[13] Because the SHS-exposed apoE$^{-/-}$ mice had lower ANT-specific activities, it is possible that chronic hypercholesterolemia imparts higher basal levels of oxidative stress in apoE$^{-/-}$ mice (supported by the increased levels of protein nitration observed in unexposed apoE$^{-/-}$ compared with C57 mice), making the ANT in these mice more sensitive to SHS exposure. Because oxidative stress purportedly increases with age, we suspect that ANT activity in older animals would be more susceptible to the effects of SHS exposure or hypercholesterolemia.

Because intermediate to advanced atherosclerotic lesions are typically not observed even in apoE$^{-/-}$ mice until 15 weeks of age or older when fed the chow diet (4% fat, as in this study), the lack of significant atherosclerotic lesion development in the normocholesterolemic C57 mice exposed to SHS is not surprising. Regardless, oil red O staining did increase with SHS exposure in normocholesterolemic mice 2.1- to 3.7-fold (after 21 and 42 days of exposure, respectively). It is possible that exposure to SHS over longer periods of time will be required to observe statistically significant lesion development in the C57 mice in the absence of preexistent hyperlipidemia (work in progress).

As expected, the highest dose of SHS (30 mg/m$^3$ TSP) had the greatest impact on mtDNA damage. However, aortic mtDNA damage was significantly increased in both groups of mice, even at lower levels of SHS exposure (1 mg/m$^3$ TSP), supporting the concept that mitochondria in cardiovascular tissues are susceptible targets for SHS-mediated damage. We have previously shown that increased mitochondrial damage in endothelial and smooth muscle cells is also associated with reduced mitochondrial function in vitro.[12] Because the mitochondrion is integral for multiple cellular processes, including energy production, apoptosis, and cell signaling, significant mitochondrial damage in vivo may compromise multiple aspects of cell function.

It has become increasingly clear that inflammatory responses involving a variety of vascular factors are an important component of CVD development. Our hypothesis that mitochondrial damage is an important event in CVD is complementary to inflammatory response theories. Several studies have shown that oxidative stress is associated with increased mitochondrial DNA damage, lipid peroxidation, inhibition of electron transport, and inactivation of specific mitochondrial enzymes.[13,27–30] The mitochondrial damage associated with SHS exposure and hypercholesterolemia

reported here may compromise important metabolic processes that influence both endothelial and vascular smooth muscle cell function, key components of atherogenesis. Consequently, our findings are consistent with present theories that oxidative stress mediates CVD by causing mitochondrial damage and dysfunction in tissues, which ultimately lead to compromised energetic capacities and cell dysfunction, important early events in CVD.

## Acknowledgments

This work was supported by National Institutes of Health grants ES09318-1 and ES011172-01, the Clayton Foundation for Research, and the John Sealy Foundation (Dr Ballinger) and the California Tobacco-Related Disease Research Program (Dr Pinkerton).

## References

1. Steenland K, Thun M, Lally C, et al. Environmental tobacco smoke and coronary heart disease in the American Cancer Society CPS-II cohort. *Circulation*. 1996;94:622–628.
2. American Heart Association. *2001 Heart and Stroke Statistical Update*. Dallas, Tex: American Heart Association; 2000:1–32.
3. Celemajer DS, Adams M, Clarkson P, et al. Passive smoking is associated with impaired endothelium dependent dilation in healthy young adults. *N Engl J Med*. 1996;334:150–154.
4. Thorne S, Mullen MJ, Clarkson P, et al. Early endothelial dysfunction in adults at risk from atherosclerosis: different responses to L-arginine. *J Am Coll Cardiol*. 1998;32:110–116.
5. Valkonen M, Kuusi T. Passive smoking induces atherogenic changes in LDL. *Circulation*. 1998;97:2012–2016.
6. Tribble D, Giuliano LJ, Fortmann SP. Reduced plasma ascorbic acid concentrations in nonsmokers regularly exposed to environmental tobacco smoke. *Am J Clin Nutr*. 1993;58:886–890.
7. Reilly M, Delanty N, Lawson JA, et al. Modulation of oxidant stress in vivo in chronic cigarette smokers. *Circulation*. 1996;94:19–25.
8. Sinzinger H, Kefalides A. Passive smoking severely decreases platelet sensitivity to antiaggregatory prostaglandins. *Lancet*. 1982;2:392–393.
9. Penn A, Chen LC, Synder CA. Inhalation of steady-state sidestream smoke from one cigarette promotes atherosclerotic plaque development. *Circulation*. 1994;90:1363–1367.
10. Glantz S, Parmley W. Passive smoking and heart disease: epidemiology, physiology, and biochemistry. *Circulation*. 1991;83:1–12.
11. Glantz S, Parmley W. Passive smoking and heart disease. *JAMA*. 1995; 273:1047–1053.
12. Ballinger SW, Patterson WC, Yan C-N, et al. Hydrogen peroxide and peroxynitrite induced mitochondrial DNA damage and dysfunction in vascular endothelial and smooth muscle cells. *Circ Res*. 2000;86: 960–966.
13. Yan L-J, Sohal RS. Mitochondrial adenine nucleotide translocase is modified oxidatively during aging. *Proc Natl Acad Sci U S A*. 1998;95: 12896–12901.
14. MacMillan-Crow LA, Crow JP, Thompson JA. Peroxynitrite-mediated inactivation of manganese superoxide dismutase involves nitration and oxidation of critical tyrosine residues. *Biochemistry*. 1998;37:1613–1622.
15. Goossens V, Grooten J, De Vos K, et al. Direct evidence for tumor necrosis factor induced mitochondrial reactive oxygen intermediates and their involvement in cytotoxicity. *Proc Natl Acad Sci U S A*. 1995;92: 8115–8119.
16. Li A, Ito H, Rovira I, et al. A role for reactive oxygen species in endothelial cell anoikis. *Circ Res*. 1999;85:304–310.
17. Plump AS, Smith JD, Hayek T, et al. Severe hypercholesterolemia and atherosclerosis in apolipoprotein E deficient mice created by homologous recombination in ES cells. *Cell*. 1992;71:343–353.
18. Reddick RL, Zhang SH, Maeda N. Atherosclerosis in mice lacking apoE, evaluation of lesional development and progression. *Arterioscler Thromb*. 1994;14:141–147.
19. Allain C, Poon L, Chan C. Enzymatic determination of total serum cholesterol. *Clin Chem*. 1974;20:470–475.
20. Flohe L, Otting F. Superoxide dismutase assays. *Methods Enzymol*. 1984; 105:93–104.
21. Graham BH, Waymire KG, Cottrell B, et al. A mouse model for mitochondrial myopathy and cardiomyopathy resulting from a deficiency in the heart/muscle isoform of the adenine nucleotide translocator. *Nat Genet*. 1997;16:226–234.
22. Ischiropoulos H, Zhu L, Chen J, et al. Peroxynitrite mediated tyrosine nitration catalyzed by superoxide dismutase. *Arch Biochem Biophys*. 1992;298:431–437.
23. Gairola CG, Drawdy ML, Block AE, et al. Sidestream cigarette smoke accelerates atherogenesis in apolipoprotein $E^{-/-}$ mice. *Atherosclerosis*. 2001;156:49–55.
24. Beckman JS, Ye YZ, Anderson PG, et al. Extensive nitration of protein tyrosines in human atherosclerosis detected by immunohistochemistry. *Biol Chem Hoppe Seyler*. 1994;375:81–88.
25. Darley-Usmar V, Wiseman H, Halliwell B. Nitric oxide and oxygen radicals: a question of balance. *FEBS Lett*. 1995;369:131–135.
26. Radi R, Rodriguez M, Castro L, et al. Inhibition of mitochondrial electron transport by peroxynitrite. *Arch Biochem Biophys*. 1994;308:89–95.
27. MacMillan-Crow LA, Crow JP, et al. Nitration and inactivation of manganese superoxide dismutase in chronic rejection of human renal allografts. *Proc Natl Acad Sci U S A*. 1996;93:11853–11858.
28. Stachowiak O, Dolder M, Wallimann T, et al. Mitochondrial creatinine kinase is a prime target of peroxynitrite-induced modification and inactivation. *J Biol Chem*. 1998;273:16694–16699.
29. Bindoli A. Lipid peroxidation in mitochondria. *Free Radic Biol Med*. 1988;5:247–261.
30. Williams MD, Van Remmen H, Conrad CC, et al. Increased oxidative damage is correlated to altered mitochondrial function in heterozygous manganese superoxide dismutase knockout mice. *J Biol Chem*. 1998;273: 28510–28515.